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UNIVERSITÀ DEGLI STUDI DI TORINO

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Title

Expression of different types of [FeFe]-hydrogenase genes in bacteria isolated from a population of a bio-hydrogen pilot-scale plant

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Abstract

[FeFe]-hydrogenases are the enzymes responsible for high yield H₂ production during dark fermentation in bio-hydrogen production plants. The culturable bacterial population present in a pilot-scale plant efficiently producing H₂ from waste materials was isolated, classified and identified by means of 16S rDNA gene analysis. The **culturable part of the** mixed population consists of nine bacterial species that include non-hydrogen producers (*Lactobacillus*, *Enterococcus* and *Staphylococcus*) and several *Clostridium* that are directly responsible for H₂ production.

An extensive analysis of the expression of [FeFe]-hydrogenases in the three best producer strains was achieved by RT-PCR, covering the complete set of known genes for each species. This revealed that during H₂ production there are several different [FeFe]-hydrogenases simultaneously expressed, with genes belonging to the same phylogenetic and structural classification sharing similar transcriptional profiles.

Keywords

Bio-hydrogen, [FeFe]-hydrogenase, *Clostridium*, Bacterial consortium, Waste.

1. Introduction

The characterisation of bacterial consortia involved in H₂ production in pilot-scale and operative plants with dark fermentation is of applicative and theoretical importance. In fact, dark fermentation is recognised as a feasible method for sustainable hydrogen production combined to treatment and valorisation of waste biomasses [1, 2].

A crucial point for a biotechnological approach is that of devising methods to optimise and stabilise the microbial consortia that ensure high productivity [2, 3], as well as elucidating the biochemical pathways that can be tuned or controlled for the hydrogen production purpose. A particular interest concerning the precise mode and timing of hydrogen production is relevant for bio-hytane production, a very promising fuel that can be obtained in two-phase dark fermentation plants by suitably combining bio-hydrogen and bio-methane streams [4].

The characterisation of the bacterial populations, including both hydrogen producers and non producers, provides information that can support the improvement of hydrogen production. In fact, hydrogen producers can have very different metabolic strategies resulting in variable yields of hydrogen. The study of the consortia still lacks some crucial points, such as a complete listing and characterisation of the diversity of genes -and proteins- that in hydrogen producers are the first responsible for high hydrogen productivity, namely hydrogenases. In this respect, there are recent works on the non-hydrogen producers [2,5-8], while concerning hydrogenases diversity in strains found in dark fermentation plants the available papers focus only on few genes and proteins. In particular the diversity of [FeFe]-hydrogenases has not yet been fully investigated in the perspective of achieving optimised performances in dark fermentation plants. Thus, the study of new [FeFe]-hydrogenases, their time and mode of expression, leads to a better knowledge of the mechanisms of catalysis for improved biotechnological applications [9-15].

[FeFe]-hydrogenases are the best performing hydrogen producing biocatalysts [16-21], with turnover frequency of up to 10^4 s^{-1} . These enzymes have a modular structure: in addition to the catalytically active domain (named H-domain), several other accessory domains may be present [17, 22]. Moreover, the functional enzymes can be monomeric, dimeric, trimeric or tetrameric, having the various domains on the same polypeptide chain or separated over one or more subunits. The detailed analysis of the sequences of [FeFe]-hydrogenases allows the definition of different groups and subgroups on the basis of the phylogenesis and domains composition [23].

The main feature common to all known [FeFe]-hydrogenases is the highly conserved part of the H-domain surrounding the active site. This region is characterised by three signature sequences containing the four cysteines that coordinate the active site and are named motifs L1 (TSCCPxW), L2 (MPCxxKxxE) and L3 (ExMACxxGCxxG) [23].

The detailed analysis of the [FeFe]-hydrogenases observed into the genus *Clostridium* revealed a very large number of enzymes with an exceptional diversity [23, 24]. Even within the same strain up to 8 genes encoding for different [FeFe]-hydrogenases can be detected, all belonging to different groups, thus suggesting different roles, interactions with several physiological redox partners and a complex regulation of the biological function of each enzyme.

So far, the high diversity of [FeFe]-hydrogenases has been studied both in natural environments [25-27] and several pilot scale plants for H_2 production [28-30].

Here the [FeFe]-hydrogenases diversity is investigated in the bacteria isolated from a pilot scale plant for H_2 production fed with waste materials that was previously described [31]. The high efficiency observed suggested the involvement of highly efficient H_2 producing microorganisms and highly efficient hydrogenases.

With the aim of characterising the vital form of the bacterial species present in the plant, the **culturable part of the** population present at the time of highest percentage in hydrogen produced was isolated, giving **specific, although partial**, insights into the composition of the consortium responsible for the wastes degradation and H₂ production. Extensive RT-PCR analysis of the [FeFe]-hydrogenases expressed by the isolated hydrogen-producing strains is presented.

The novelty of this work is that RT-PCR was performed covering the complete set of known [FeFe]-hydrogenase genes as available in databases and classified in recently published literature [21, 23, 24], while several previous studies in various microorganisms have focused on a single [FeFe]-hydrogenase gene only [29, 32-38].

2. Materials and Methods

2.1. Pilot-scale plant set-up and operation

Dark anaerobic fermentation was carried out in a continuous stirred tank reactor (CSTR) of 35 litres, equipped with pH, oxidation/reduction potential and temperature control system. The temperature control was done through the inlet of hot water in the heating jacket; to avoid stratification, the pilot reactor was provided with an external recirculating system through a peristaltic pump.

The substrate used for the fermentative process was a mixture composed of 80% of fruits and vegetables wastes and 20% of manure (20 mg/mL total suspended solids-2% total suspended solids), supplemented with a micronutrient medium previously described [31].

The inoculum used (10% v/v) was a digested sludge coming from the municipal wastewater treatment plant of Torino, chemically pretreated with HCl for 24 hours at pH 3 to inhibit the methane forming bacteria and enrich the H₂ producing flora [39-41].

The operating volume was 25 litres, nitrogen gas was injected till no oxygen was detected to create adequate anaerobic conditions, pressure inside the reactor was set at values around 20-30 mbar. The experiment was carried out at pH 5-6 and temperature between 25 and 30°C.

The volume of gas produced was measured through a drum-type gasmeter (Dr.-Ing. Ritter Apparatebau GmbH & co.) whereas the gas composition (H₂ and CO₂ mainly) was determined by using a micro gas chromatograph (Varian, CP4900).

2.2. Isolation of culturable bacteria

Bacteria were isolated from a sample of the digestate present in the plant during its operation.

Un-treated samples were diluted and directly plated on the rich non selective “Clostridial nutrient” medium (Sigma-Aldrich) agar plates. Alternatively, to select for spore-forming microorganisms, the sample was heat-treated at 80°C for 10 minutes prior to dilution. Plates

were incubated under anaerobic conditions into “Anaerogen” bags (Oxoid) at 37°C over night.

Forty single and isolated colonies with different morphology were randomly selected and pure cultures were obtained by an additional passage on plate.

Morphological characterisation was obtained using Gram staining. Discrimination between obligate and facultative anaerobes was obtained detecting the growth on plates incubated under anaerobic or aerobic conditions.

2.3. 16S rDNA amplification, RFLP analysis and sequencing

Genomic DNA was extracted from colonies of each isolate by rapid freeze-thawing in sterile water [42]. 16S rDNA gene was amplified from genomic DNA by PCR using the proof-reading polymerase “KOD Hot Start DNA polymerase” (Merck Millipore) and the two universal primers 27F (5'-AGAGTTTGATYMTGGCTCAG) and 1492R (5'-TACGGYTACCTTGTTACGACT) [42].

Restriction fragment length polymorphism (RFLP) analysis was performed using the four restriction endonucleases *AluI*, *HaeIII*, *HhaI* and *TaqI* (Thermo Scientific) that were previously reported to have a high discriminatory power [43]. The amplified 16S rDNA fragment was digested following the manufacturer's instructions. DNA fragments were separated by electrophoresis on 1.5% w/v agarose gels in 1x TAE stained with SYBR® Safe (Invitrogen) using “PerfectSize DNA Molecular Weight 100 bp XL Ladder” (5Prime) as a reference.

After classification, two isolates per each group were identified by sequencing the entire PCR fragment from an external company (Eurofins MWG Operon, Germany). The sequences were compared with the NCBI database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Exact matches were found for each sequence with 100% identity.

2.4. Quantification of H₂ production yield of the strains

Pure cultures of each species were grown in sealed glass vials under an anaerobic argon atmosphere at 37°C with shaking. The vial contained a medium that was previously reported [42] containing 100 mM potassium phosphate, 17 g/L tryptone, 3 g/L peptone papaic digest of soybean, 10 g/L glucose, initial pH 7.0.

The gas phase was sampled with a SampleLock Gastight syringe (Hamilton) and analysed by gas chromatography, at least in three replicates. The gas chromatographer (Agilent Technologies 7890A) was equipped with purged packed inlet, Molesieve 5A column (30 m, ID 0.53 mm, film 25 mm) and thermal conductivity detector; argon was used as carrier gas.

2.5. RT-PCR

Samples were taken from the pure cultures at the time points shown in Figure 4. Total RNA was extracted and purified using the kit “Total RNA isolation NucleoSpin® RNA II” (Macherey-Nagel). Since genomic DNA contamination was often observed, the total RNA was also subjected to an additional DNase I treatment (Sigma-Aldrich).

Reverse transcription of total RNA into cDNA was achieved with random hexamers using “Maxima H Minus First Strand cDNA Synthesis Kit” (Thermo Scientific) after normalisation of the concentration.

Specific primers were designed for each target gene using the tool “eprimer3” (<http://emboss.bioinformatics.nl/cgi-bin/emboss/eprimer3>) and the genome sequences deposited in the NCBI database: *Clostridium beijerinckii* (NC_009617.1), *Clostridium butyricum* (PRJNA54843) and *Clostridium perfringens* (NC_008261.1). Specificity was tested by checking the sequence of each primer against the sequence of the other [FeFe]-hydrogenase genes; moreover the PCR products have been sequenced showing the specific amplification of the target gene only.

Detection of each transcript was obtained by PCR using “KOD Hot Start DNA polymerase” and the following conditions: 95°C for 2 minutes, 38 cycles of 95°C for 20 seconds, 53°C for 10 seconds, 70°C for 14 seconds.

During RT-PCR, opportune controls were always performed as follows: genomic DNA (positive control), no template (NTC) and no reverse transcriptase (RT-). The 16S rDNA gene was also included in the RT-PCR analysis to validate the quality of the cDNA and to be used as a reference for the semi-quantitative estimate of the other transcripts.

3. Results

3.1. Hydrogen production via dark anaerobic fermentation in a CSTR reactor.

The pilot scale plant for H₂ production from waste materials, designed and built within the “BioEnergy Lab” of the “Environment Park” in Torino, Italy, has already been described [31].

This plant showed to be efficient in the production of H₂ from a number of different wastes; the biogas recovered could be directly used to generate electricity [31].

For the present study the plant was fed with a mixture of vegetable wastes from food markets supplemented with 20% manure. The inoculum was composed of sludge from a wastewater treatment plant that was pre-treated with HCl to inhibit the methanogenic microorganisms.

After a lag time of 30h and a batch phase of 60h, the plant was operated with a hydraulic retention time (HRT) of 30h. The production of hydrogen and CO₂ was followed as a function of time. Hydrogen percentage ranged between 20 and 50%, with a maximum value of 54-55% (Figure 1). This value is interestingly higher in comparison to previously published percentages, typically in the range 20-40% [44, 45].

The average hydrogen production rate was 0.9 L/h with a maximum yield of 72.6 mLH₂/g volatile suspended solids and a productivity of 0.72 (LH₂) (L culture)⁻¹ day⁻¹. No methane production was observed, demonstrating that acid pre-treatment of inoculum is an effective method to avoid methanogenesis during fermentation.

A sample of the digestate was taken (Figure 1) at the maximum hydrogen production percentage (54% of hydrogen, 46% of carbon dioxide) with a close-to-average hydrogen production rate (0.9 L/h) for the isolation of the culturable bacteria.

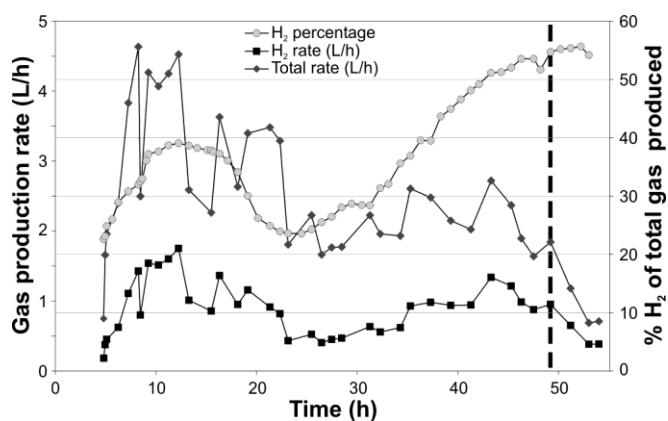


Figure 1 - Amount and rate of hydrogen production in the pilot plant. The left vertical axis is referred to the gas production rate; the right axis is referred to the percentage of hydrogen on total gas produced (the main other gas is represented by CO₂). The dashed vertical line indicates the time when the sampling of the digestate was performed for studies of microbial flora isolation. (1 column fitting).

3.2. Isolation and characterisation of the culturable bacterial population.

All the isolates were classified on the basis of cell morphology after Gram staining and the ability to grow also in the presence of oxygen, thus discriminating the obligate from the facultative anaerobes (Table 1).

Subsequently, the isolates were classified in more details on a molecular basis, by means of 16S rDNA gene restriction fragment length polymorphism (RFLP) analysis which allowed the subdivision of all the isolated strains into 9 different groups (Figure 2).

Each group was then identified by sequencing the entire 16S rDNA gene amplicon and searching against the NCBI database; exact matches were found per each sequence with 100% identity. The 9 groups were identified as 9 different bacteria (Table 1) and resulted to be four species of the genus *Clostridium*, three species of the genus *Enterococcus*, one species of the genus *Lactobacillus* and one species of the genus *Staphylococcus*.

The ability to produce H₂ was assayed to investigate the direct involvement in H₂ production of each species (Table 1). This showed that only the four species of the genus *Clostridium* are

able to produce H₂. Among them, the bacterium showing the highest hydrogen production yield is *C. beijerinckii* SM10, followed by *C. butyricum* SM32, *C. perfringens* SM09 and *C. bifermentans* SM11.

ID	Species	Strain	Cell morphology	Spores	Anaerobe	mL H ₂ / g glucose
1	<i>Clostridium beijerinckii</i>	SM10	bacilli, Gram +	-	Obligate	247.2 ± 9.6
2	<i>Clostridium butyricum</i>	SM32	bacilli, Gram +	+	Obligate	136.3 ± 4.6
3	<i>Clostridium perfringens</i>	SM09	bacilli, Gram +	-	Obligate	130.6 ± 3.0
4	<i>Clostridium bifermentans</i>	SM11	bacilli, Gram +	+	Obligate	97.1 ± 9.2
5	<i>Lactobacillus plantarum</i>	SM21	bacilli, Gram +	-	Facultative	0
6	<i>Enterococcus sp.</i>	SM01	cocci, Gram +	-	Obligate	0
7	<i>Enterococcus devriesei</i>	SM03	cocci, Gram +	-	Obligate	0
8	<i>Enterococcus sp.</i>	SM08	cocci, Gram +	-	Facultative	0
9	<i>Staphylococcus hominis</i>	SM17	cocci, Gram +	-	Facultative	0

Table 1 - Summary of the **culturable** strains identified in the pilot plant. Each species was identified by 16S rDNA gene sequencing. **Cell morphology and the presence of spores were determined by Gram staining of the isolated strains. The ability to grow in the presence of oxygen allowed discrimination between obligate and facultative anaerobes. The ability of each species to produce H₂ was determined. Strains 1 to 4 were isolated from heat-treated samples, while strains 5 to 9 were isolated from un-treated samples.**

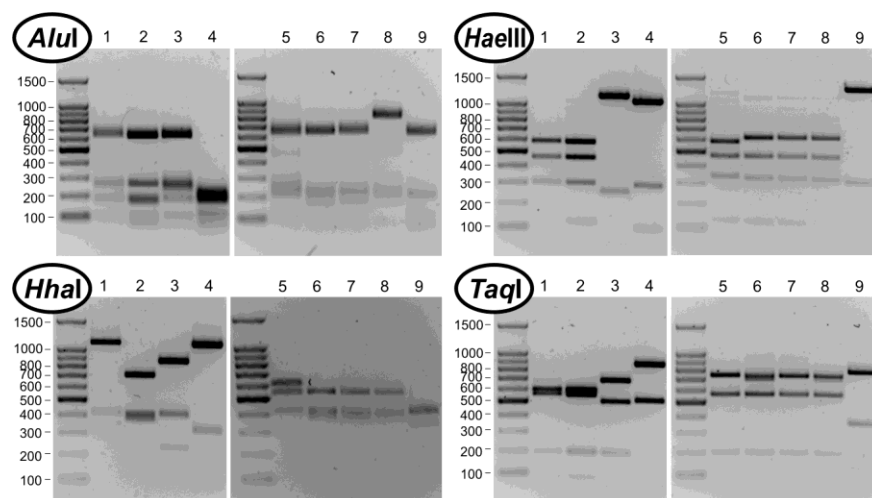


Figure 2 - 16S rDNA gene RFLP analysis summary of the nine strains isolated from the pilot plant. The 16S rDNA gene was amplified by PCR and digested with the following restriction endonucleases: *AluI* (cuts AGCT), *HaeIII* (cuts GGCC), *HhaI* (cuts GCGC) and *TaqI* (cuts TCGA). (1.5 column fitting).

3.3. [FeFe]-hydrogenase genes expression during H₂ production.

Fermentative hydrogen production in the genus *Clostridium* requires the catalytic activity of hydrogenases, redox enzymes that use protons as the final electron acceptors in the cellular energy metabolism [23, 46]. Analysis of the known *Clostridium* species genomes revealed that each species does not possess a single gene encoding for this enzyme but many genes, usually between 2 and 8, encoding for different hydrogenases [23, 24]. Most of the genes are annotated to encode for [FeFe]-hydrogenases, usually involved in H₂ evolution, and only few of them encode for [NiFe]-hydrogenases, usually involved in H₂ uptake. Thus, the genome sequence analysis revealed a complex framework with many genes and enzymes potentially involved in H₂ production that so far is supported by very few experimental data concerning the physiological role of the various hydrogenases.

The genomes of the three species with the highest hydrogen production yield isolated here have been previously sequenced: *C. beijerinckii* NCIMB 8052, *C. butyricum* 5521 and *C. perfringens* ATCC 13124. Altogether, they have annotation of 14 genes encoding for different [FeFe]-hydrogenases (Figure 3) that belong to different modular structure and phylogenetic classification [23, 24]. Most genes encode for monomeric enzymes, while only three of them encode for heterotrimeric enzymes. All the gene products are predicted to be cytoplasmic, with the exception of that belonging to cluster A3 (structure TR(M2)). It has to be noted that only *C. beijerinckii* has also a gene annotated to encode for a [NiFe]-hydrogenase.

Interestingly, most of these genes or enzymes have never been studied into details before. *C. butyricum* and *C. perfringens* have a gene encoding for an [FeFe]-hydrogenase of the M3 structure; enzymes from this group (CpI and CaHydA) are known to be directly involved in H₂ production and they have been previously characterised in details in *C. pasteurianum* [47-50] and *C. acetobutylicum* [20, 33, 51, 52] respectively. On the contrary, *C. beijerinckii* lacks enzymes of this well known type.

In order to study the **transcriptional levels** of each of these genes in hydrogen production in the three strains isolated here, their expression at mRNA level was studied by RT-PCR during the growth of pure cultures.

Interestingly, H₂ evolution occurs mainly during the exponential growth phase in *C. beijerinckii* SM10 and *C. butyricum* SM32, while it is mainly observed during the late exponential and stationary phase in *C. perfringens* SM09 (Figure 4), thus suggesting important differences in the role of H₂ production in the metabolism. On the basis of the relationship between growth and H₂ production, time points were selected for the study of [FeFe]-hydrogenase expression.

Specific primer couples targeting each gene were designed (Table 2) on the basis of the sequenced genomes and tested with the genomic DNA of *C. beijerinckii* SM10, *C. butyricum* SM32 and *C. perfringens* SM09. PCR fragments with the expected length could be amplified; **DNA sequencing of the PCR product** demonstrated the specificity and efficiency of the amplification and also the presence of each gene in the genome of the strains isolated from the plant (data not shown).

RT-PCR (Figure 3) showed that transcripts of all the [FeFe]-hydrogenases analysed could be detected. Moreover, the **transcriptional profiles were** interesting: most genes were constitutively expressed and only few of them showed a modulation.

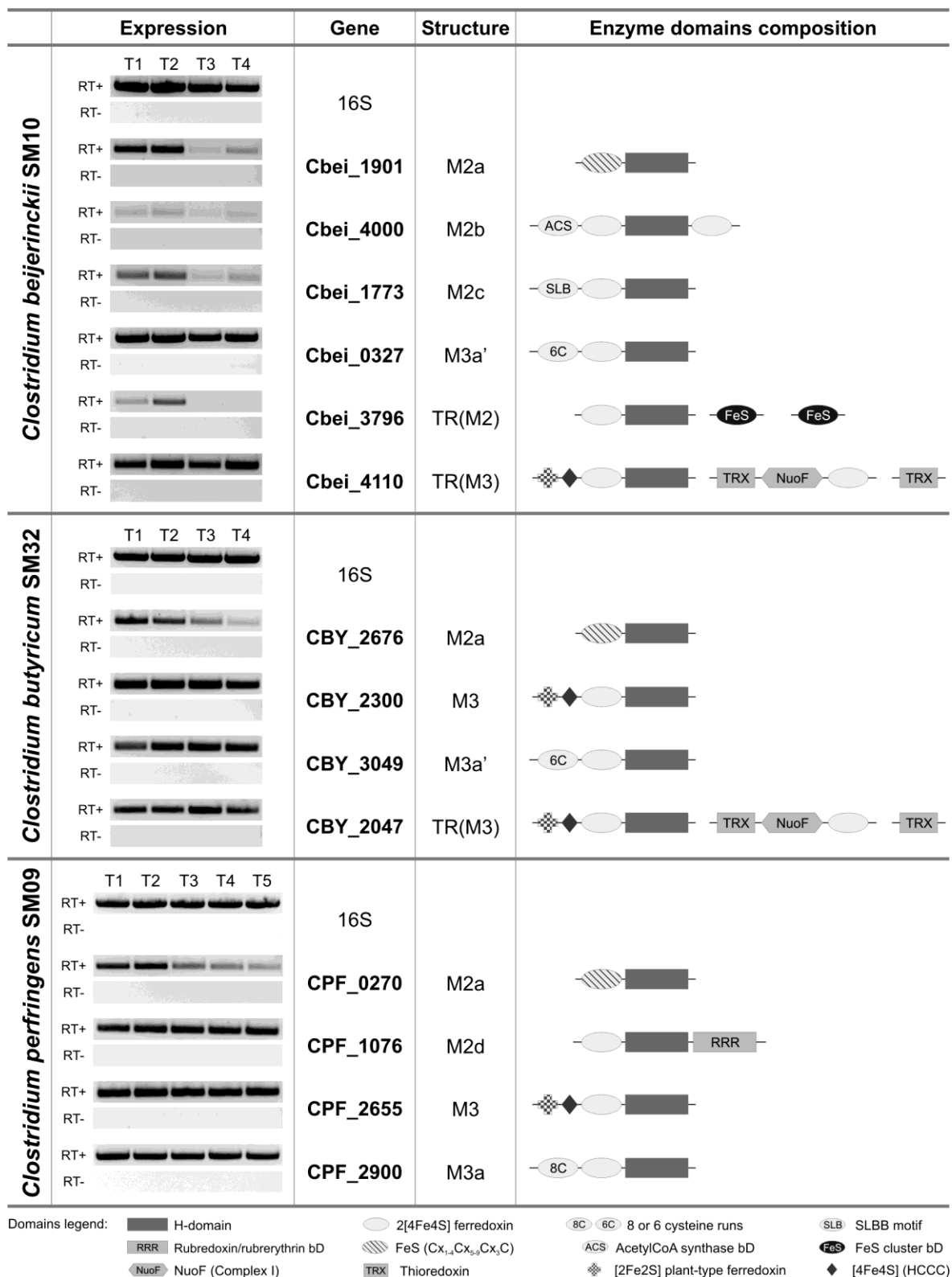


Figure 3 - [FeFe]-hydrogenase genes expression in *C. beijerinckii* SM10, *C. butyricum* SM32 and *C. perfringens* SM09. The expression was studied by RT-PCR, using the 16S gene as a

reference. The modular structure classification and the domains composition refer to the enzyme in accordance with the classification proposed before [23]. (2 column fitting).

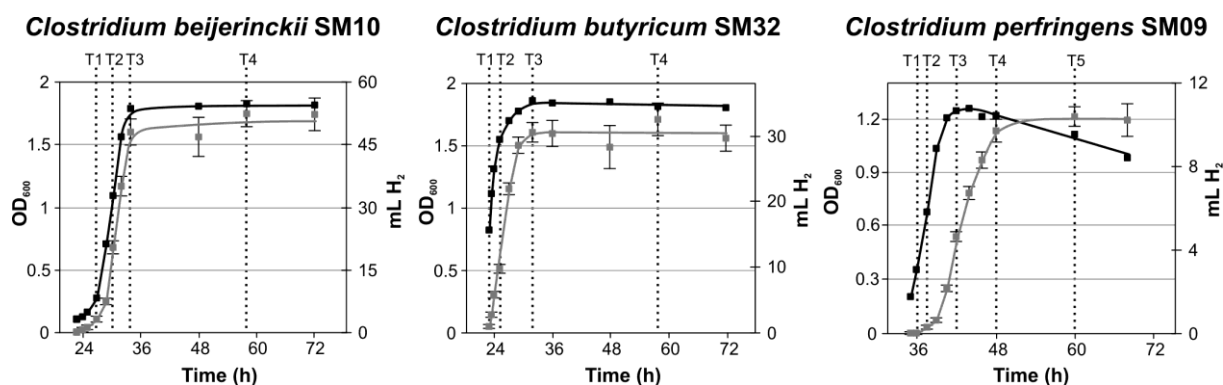


Figure 4 - Hydrogen production by pure cultures of *Clostridium beijerinckii* SM10, *C. butyricum* SM32 and *C. perfringens* SM09. The growth was monitored by measuring the OD₆₀₀ (black line). The cumulative H₂ production (grey line) was monitored by gas chromatography. The time points selected for the study of [FeFe]-hydrogenase genes expression are marked by dotted lines. (2 column fitting).

Gene	Forward	Reverse	Length
Cbei_1773	GTATTGGATGCGGAGCTTGT	TCCTGGTTCAAAGCCAAAAG	260 bp
Cbei_0327	TCATGTCCAACAGGTGCATT	CCGCCTGAGATCTTTACTGG	770 bp
Cbei_1901	CGGTTTCACCTATGGTTGCT	TCGGCTGATTCCCTACCTTG	484 bp
Cbei_4110	CAGCGGAAGTATGTGCAATG	CCGCACCAGCTATACATCCT	381 bp
Cbei_3796	AGCCAGTGTTCAGCTTTT	TGCAGCTTCCATTACACCAC	683 bp
Cbei_4000	GGCAGCAGCTCTTTTGGTA	TTTATGCACCCTCCGTTACA	279 bp
CBY_3049	TCACCAATGGTTGCTACAGG	ACCTATTGCAAACCCCTTC	246 bp
CBY_2300	ATGGGTTAGGCAAGCAGAAA	GCTGGATCTGCCTTCTCATC	312 bp
CBY_2047	CCAGCAGTAAGGTTGCACT	CCAAACATTTCCATTGGTGA	401 bp
CBY_2676	CCTATGTGGGTTGGCATGTT	TAAGGGTTTTGGACCACCA	514 bp
CPF_2655	AAGGCAAGAGCTCACAAACC	TAATGCATCAACTGGGCAAG	281 bp
CPF_1076	GAGTCCAGCTTCAGCCTTG	CTCTCCTTCTGCCCCATGTA	331 bp
CPF_0270	TCTTGCTGTTGCCCTATGTG	CCTCCAACACATCCCATACC	500 bp
CPF_2900	ATTCAATGTGGAGCCTGTGT	CTTGCAAGCCATTCTTCT	769 bp

Table 2 – List of the primers designed for RT-PCR with the size of the amplified fragment.

4. Discussion

The culturable **part of the** bacterial population present in a bio-hydrogen pilot-scale plant at the maximum H₂ production **percentage** was characterised, revealing the presence of microorganisms belonging to the genera *Clostridium*, *Lactobacillus*, *Enterococcus* and *Staphylococcus*.

The involvement of *Clostridium* species in H₂ production is well known and they are reported to be able to use several waste matrices [1, 53]. Various strains of *C. beijerinckii* were isolated from several sources but, interestingly, the hydrogen production yield of the strain isolated here is higher than those previously reported [54-56]. Moreover *C. beijerinckii* is one of the major producers of butanol, an important solvent that can be used as biofuel [57], thus suggesting another possibility to recover simultaneously different valuable products from wastes.

In the culturable part of the bacterial population, the presence of several species that are not directly involved in H₂ production reveals that a complex consortium of microorganisms with different roles was spontaneously selected in the pilot plant, giving place to efficient matrix degradation and H₂ production.

Lactobacillus plantarum has been found in a wide range of natural and artificial fermentation processes, in particular in plant-derived matrices [58]. It is a very versatile microorganism that produces large amount of lactic acid and causes acidification of the fermentation medium [59], thus favouring the hydrogen producing microorganisms versus the methanogenic ones. Also *Enterococcus* species are found in fermentation processes, including food products, and have a metabolism that results in lactic acid production and acidification [60]. Interestingly, a consortium for hydrogen production between *C. butyricum* and *Enterococcus saccharolyticus* was previously reported [61].

It is also interesting to consider that *Lactobacillus plantarum* produces various bacteriocins that can inhibit the growth of several microorganisms, including *Clostridium*, *Enterococcus* and *Staphylococcus* species; importantly, many strain-to-strain differences have been reported [59, 62-64]. Also various *Enterococcus* species are reported to produce bacteriocins [60, 65].

In this case, the coexistence of *Lactobacillus plantarum* and *Enterococcus* species with various *Clostridium* species suggests that the strains isolated here interact favourably between them and, conversely, that the bacteriocins potentially produced by *L. plantarum* [59, 62] and *Enterococcus* sp. [60] might act to select the specific strains within the consortium.

The involvement of [FeFe]-hydrogenase in hydrogen production in *Clostridium* is known [23], but the specific investigation of the role of different [FeFe]-hydrogenases has never been performed before. Here, the study of the expression at the mRNA level by RT-PCR was performed on pure cultures of the isolated *C. beijerinckii* SM10, *C. butyricum* SM32 and *C. perfringens* SM09 during the growth, showing that all the known genes (6, 4 and 4, respectively) are expressed. The constitutive expression of [FeFe]-hydrogenase genes at the mRNA level is not simple to interpret, especially for the time points where the amount of accumulated H₂ does not change, suggesting a post-transcriptional down-regulation of the functionality or an equilibrium between H₂ evolution and uptake.

C. beijerinckii SM10 shows the most complex behaviour, with two genes constitutively expressed in all the four time points: Cbei_0327 (M3a' structure) and Cbei_4110 (TR(M3 structure)).

Interestingly, the other four genes of *C. beijerinckii* are variants of the M2 modular structure (M2a, M2b, M2c and TR(M2)) and they share a common behaviour in terms of transcripts: they are all down-regulated during the stationary phase.

Cbei_4110 was previously reported to be expressed in *C. beijerinckii* RZF-1108 [38] but surprisingly, in a recent genome-wide transcriptional analysis of *C. beijerinckii* NCIMB 8052, there is no mention of any hydrogenase genes expression levels [66].

C. butyricum SM32 has three genes constitutively expressed: CBY_2300 (M3 structure), CBY_3049 (M3a' structure) and CBY_2047 (TR(M3) structure), while CBY_2676 (M2a structure) is gradually down regulated.

CBY_2300 belongs to the M3 structure (group A2) and the encoded protein has high sequence similarity with the well characterised CpI and CaHydA (67.5% and 66.2% identity, respectively); CBY_2300 expression was previously reported both in experiments with pure [36] and mixed [29, 34] cultures. Moreover, CBY_2300 has already been demonstrated to encode for a functional [FeFe]-hydrogenase enzyme that can be heterologously expressed both in *E. coli* and *C. acetobutylicum* [37].

Cbei_4110 and CBY_2047 share a common transcriptional regulation and a high sequence identity (63.8%). The enzymes encoded by these genes are considered to include also other two subunits found in the same operon [23], resulting to be similar to the so-called heterotrimeric bifurcating hydrogenases, recently identified in other anaerobic species [67-69]. These enzymes can synergistically couple the oxidation of NADH with the oxidation of ferredoxin, yielding H₂ as a final product, and allowing a fine tuning of the electron fluxes within the cell [67]; nevertheless, it must be noticed that bifurcating [FeFe]-hydrogenases were also reported to be able to catalyse the inverse reaction of H₂ uptake coupled to ferredoxin and NADH reduction [68]. Thus the exact physiological role of Cbei_4110 and CBY_2047 remains to be experimentally demonstrated.

Despite the different kinetics of H₂ production, *C. perfringens* SM09 genes are similar to those of *C. butyricum* SM32 in terms of classification and they share a similar transcriptional

profile: CPF_2655 (M3 structure), CPF_2900 (M3a structure) and CPF_1076 (M2d structure) are constitutively expressed, while CPF_0270 (M2a structure) is gradually down regulated. Like CBY_2300, also CPF_2655 belongs to the M3 structure (group A2) and the encoded protein has high sequence similarity with the well characterised CpI and CaHydA (68.8% and 68.7% identity, respectively). The enzyme was never studied, but the gene was characterised into details: it is always expressed during the growth on glucose, in accordance with the results presented here, and found into two different transcripts [32]. Moreover, the disruption of CPF_2655 abolishes H₂ productivity in *C. perfringens* str. 13, demonstrating its direct involvement in H₂ production [32].

It is to be noticed that genes belonging to the same modular structure and same phylogenetic group share similar transcriptional **levels** even in different microorganisms, such as Cbei_4110 and CBY_2047 (TR(M3) structure); CBY_2300 and CPF_2655 (M3 structure); Cbei_0327, CBY_3029 and CPF_2900 (M3a and M3a' structures); Cbei_1901, CBY_2676 and CPF_0270 (M2a structure).

5. Conclusions

The culturable **part of the** bacterial population present in a bio-hydrogen pilot-scale plant at the maximum H₂ production percentage was isolated and characterised, revealing that a complex consortium of bacteria belonging to the genera *Clostridium*, *Lactobacillus*, *Enterococcus* and *Staphylococcus* was spontaneously selected within the plant, participating to efficient matrix degradation and H₂ production.

The characterisation of the isolated *Clostridium* strains and the simultaneous study of the expression of all the [FeFe]-hydrogenase genes for each strain, as presented here, brings a novel contribution to the field and gives the first insights on the complex metabolic network of the [FeFe]-hydrogenase function.

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